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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Hiroaki Yamamoto
Serial No. : 09/305,390
Filed : May 5, 1999
Title : METHOD FOR PRODUCING OPTICALLY ACTIVE 4-HALO-3-HYDROXYBUTYRIC ACID ESTER

Art Unit : 1652
Examiner : R. Hutson

Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF HIROAKI YAMAMOTO UNDER 37 C.F.R. § 1.132

1. I am the sole inventor of the above-identified U.S. patent application.

2. I am making this Declaration to provide relevant facts in support of the patentability of the subject matter claimed in the patent application.

3. I have read and understood the outstanding Office Action mailed March 15, 2000.

4. I understand that the Examiner contends that claims 7-10, 12, 14, and 19-22 are not enabled for the full scope of the claims. Rather, the Examiner maintains that the claimed methods are enabled only for use of the acetoacetyl-CoA reductase from *Ralstonia eutropha*, as described at pages 31-34 and 36-39, Examples 12-16 and 21-24 of the specification.

5. To demonstrate that other acetoacetyl-CoA reductases can be used in the claimed methods in accordance with the teachings of the specification, I have cloned and expressed acetoacetyl-CoA reductase from *Zoogloea ramigera* and used this enzyme to asymmetrically reduce a 4-halo-acetoacetic acid ester, as described below.

6. The *Zoogloea ramigera* gene encoding acetoacetyl-CoA reductase is described in Peoples et al., Mol. Microbiol. 3:349-357 (1989). Based on this published gene

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I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231.

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sequence, two PCR primers (ZRAR-AT1, ZRAR-TA1) flanking the protein-coding nucleotide sequence were synthesized:

ZRAR-AT1: GCGTCATGAGTCGTGTAGCATTGGTAACGGGG

ZRAR-TA1: TGGTCTAGATTAGACGAAGAACTGGCCGC

Chromosomal DNA was isolated from *Zoogloea ramigera* DSM 287 strain using standard procedures. Strain DSM 287 is identical to strain I-16-M identified in the specification at page 14, line19. This strain is available from a number of publically available sources, such as the German Collection of Microorganisms and Cell Cultures, which is an International Depository Authority under the Budapest Treaty for Biological Deposits. PCR was performed using primers ZRAR-AT1 and ZRAR-TA1 and the chromosomal DNA as a template, following the procedures described at pages 31-32, Example 12 of the specification, except that the PCR product was digested with restriction enzymes BspHI and XbaI and ligated to vector pSE420D (page 34, Example 17 of the specification) digested with NcoI and XbaI. The resulting plasmid was termed pSE-ZRR1. A second expression plasmid, pSG-ZRR1, was obtained by digesting the above-mentioned PCR fragment with BspHI and XbaI and ligating the PCR fragment to the *Bacillus subtilis* glucose dehydrogenase expression plasmid pSE-BSG1 (pages 34-35, Example 18 of the specification) digested with NcoI and XbaI.

Analysis of the nucleotide sequence of the enzyme-encoding inserts in pSE-ZRR1 and pSG-ZRR1 indicated that the coding sequence was identical to the sequence published in Peoples et al., except for a change of GC to CG at nucleotide positions 123 and 124 (nucleotide positions are numbered from the first nucleotide of the start codon).

7. *Escherichia coli* JM109 was transformed with either pSE-ZRR1 and pSG-ZRR1, inoculated into 7 ml of an LB culture medium containing ampicillin, and cultured for 12 hours at 28°C. IPTG was then added to the culture to 0.1 mM, and the bacteria were cultured for an additional 4 hours at 30°C to express acetoacetyl-CoA reductase (pSE-ZRR1) or acetoacetyl-CoA reductase and glucose dehydrogenase (pSG-ZRR1). The culture medium was centrifuged to collect the cells, which were then suspended in 0.5 ml of cell disruption buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.02% 2-mercaptoethanol, 2 mM PMSF, 10% glycerol, and 0.5 M NaCl) and disrupted by

sonication. The cell-free supernatant obtained by centrifuging the disrupted cell mixture was used for assaying enzyme activity, as described below.

8. Enzyme reactions were performed in a 1 ml reaction mixture containing 100 mM potassium phosphate buffer (pH 6.5), 0.2 mM NADPH, 20 mM ethyl 4-chloroacetoacetate (ECAA), and acetoacetyl CoA reductase extract. Enzyme activity was monitored by measuring the decrease in absorbance at 340 nm as NADPH was consumed in the reaction. One unit was defined as the activity necessary to catalyze the consumption of 1 μmol NADPH in 1 minute. Protein concentrations were measured using the Bradford method (kit from Bio-Rad). Specific activity was defined as enzyme activity per 1 mg of protein. Another reaction was performed as described immediately above, except that ethyl acetoacetate (EAA) replaced ECAA as the substrate. Enzyme activity was measured using a reaction solution containing 100 mM Tris-HCl (pH 8.0), 0.2 mM NADH, 0.2 mM acetoacetyl-CoA, and acetoacetyl CoA reductase extract.

The ability of the *Z. ramigera* reductase to oxidize (S)- or (R)-ethyl 4-chloro-3-hydroxybutyrate (SECHB and RECHB, respectively) was measured in a reaction solution containing 100 mM Tris-HCl (pH 8.0), 2.5 mM $NADP^+$, 20 mM SECHB or RECHB, and acetoacetyl CoA reductase extract.

9. The results are summarized in Tables 1 and 2.

Table 1. ECAA reducing activity and glucose dehydrogenase activity of transformed bacteria

Plasmid	GlcDH (U/mg)	ECAA-R (U/mg)
pSE-ZRR1	0.005	0.665
pSG-ZRR1	5.59	0.824

GlcDH: glucose dehydrogenase activity
ECAA-R: ethyl 4-chloroacetoacetate reducing activity

Table 2. Substrate specificity of acetoacetyl-CoA reductase

Buffer	Coenzyme	Substrate	Specific activity U/mg	Relative activity %
KPB, pH6.5	0.2 mM NADPH	20 mM ECAA	0.824	35.2
	0.2 mM NADH	20 mM ECAA	0.000	0
		20 mM EAA	0.316	13.5
Tris-HCl, pH8.0	2.5 mM NADP+	20 mM RECHB	0.002	0.1
		20 mM SECHB	0.004	0.2
Tris-HCl, pH8.0	0.2 mM NADPH	0.2 mM AcAcCoA	2.341	100
	0.1 mM NADPH	0.02 mM AcAcCoA	0.010	0.4

ECAA: ethyl 4-chloroacetoacetate
EAA: ethyl acetoacetate

RECHB: (R)-ethyl 4-chloro-3-hydroxybutyrate
SECHB: (S)-ethyl 4-chloro-3-hydroxybutyrate
AcAcCoA: acetoacetyl-CoA

10. Extract from induced *E. coli* transformed with pSE-ZRR1 exhibited an ECAA reducing activity that is not seen in parent *E.coli* JM109. In addition to the ECAA reducing activity, extract from induced *E.coli* transformed with pSG-ZRR1 efficiently expressed glucose dehydrogenase (Table 1). The substrate specificity also was examined, using the activity of the enzyme against acetoacetyl-CoA substrate as 100% relative activity (Table 2). The relative activity against the ECAA substrate was 35.2%, and the relative activity against the EAA substrate was 13.5% (Table 2). In addition, NADH did not function as a coenzyme, and the enzyme did not have substantial activity against substrates RECHB and SECHB (Table 2). These results for the *Z. ramigera* enzyme are similar to what was observed for the acetoacetyl CoA reductase from *Ralstonia eutropha* (page 33, Table 3 of the specification).

11. To determine the capacity of the *Z. ramigera* enzyme to asymmetrically reduce ethyl 4-chloroacetoacetate, cell-free extract from induced pSG-ZRR1-transformed *E. coli* was incubated in reactions as specified in Table 3.

Table 3. Ethyl 4-chloro acetoacetate reducing reaction by acetoacetyl-CoA reductase

		Reaction conditions											
			ECAA									ECHB	
	No.	KPB mM	%	M	Glc eq.	$NADP^+$ mM	ECAA-R U/mL	GlcDH U/mL	Total mL	ECAA g/L	ECHB g/L	ee(%)S	Yield (%)
1% ECAA	1	500	1.0	0.061	2.0	1.0	0.5	3.4	2	0.00	9.52	>99%	95.2
2% ECAA	2	500	2.0	0.122	2.0	1.0	0.5	3.4	2	0.00	18.58	99.5	92.9

ECAA: ethyl 4-chloroacetoacetate
Glc: glucose
GlcDH: glucose dehydrogenase
ECAA-R: ethyl 4-chloroacetoacetate reductase
ECHB: ethyl 4-chloro-3-hydroxybutyrate

The optical purity of the ethyl 4-chloro-3-hydroxybutyrate product was measured after overnight incubation at 25°C. The product was synthesized with a yield of 92% or more for ECAA substrate concentrations of 1% and 2%. The optical purity was confirmed to be extremely high (99.5%ee; Table 3). These results are similar to what was observed for the acetoacetyl CoA reductase from *Ralstonia eutropha* (page 34, Example 16 of the

specification). Amounts of ethyl (S)-4-chloro-3-hydroxybutyrate and the optical purity thereof were determined as described in the working examples of the specification.

12. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of any patent issuing from the present patent application.

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Date Hiroaki Yamamoto

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